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Changes in Lipid Composition in the Tail of *Rana catesbeiana* Larvae during Metamorphosis

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ABSTRACT—The composition of simple lipids and phospholipids obtained from the larval tails in *Rane catesbeiana* at Taylor and Kollros (TK) stage V, X, XX, XXI, and XXII-XXIII were analyzed to clarify their relationship with the regressive process of tails during metamorphosis in this frog. The weight percentage of total simple lipid to total lipid (TL) was about 33% at TK stages V and X and increased gradually to about 70% at TK stages XXII-XXIII. That of total phospholipid to TL was about 66% at TK stages V and X decreased gradually to about 28% at TK stages XXII-XXIII. The weight percentage of free fatty acid (FFA) and triglyceride (TG) to TL were about 12-15% and 4% at TK stages V and X, and increased gradually to about 38% and 13% at TK stages XXII-XXIII, respectively. Those of the other simple lipids, i.e., cholesterol and cholesterol ester did not change much during metamophosis. The weight percentage of phospholipid classes, i.e., phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin and lysophosphatidylcholine to total phospholipid also did not significantly change throughout metamorphosis. The resulting increase in the weight percentage of total simple lipid to TL was due primarily to increase in FFA and TG during the metamorphic climax.

INTRODUCTION

Biochemical changes occurring during anuran metamorphosis have been reviewed by Bennett and Frieden [1], Brown [2], Frieden [3], Weber [4], Fox [5] and Yoshizato [6]. A critical review of these studies shows that although remarkable changes have been described in the ornithine-urea cycle enzymes [7], haemoglobin [8, 9], serum protein [9, 10], visual pigments [11], nucleic acids especially of liver [12] and lysosomal acid hydrolases such as acid phosphatase [13–15], comparatively very little is known of the alternations in the lipids of the anuran tadpoles during metamorphosis.

Whatever information is available on the lipids during anuran metamorphosis comes from the work of Camerio, Italian group, reviewed by Urbani [16], regarding changes during metamorphosis of *Bufo vulgaris* and *Rana esculenta*. Urbani [17] found an approximate fourfold decrease in the total body lipid of *Bufo vulgaris* during the metamorphosis period. Light and Waschek [18] studied the liver fatty acids of the tadpole and adult Rana *grylio* and reported no appreciable difference in them. Sawant and Varute [19] have reported on the lipid changes; that is, total lipids, neutral lipids and phospholipids, in the tadpole *Rana tigrina* during development. Recently Okamura and Kishimoto [20] have reported on qualitative and quantitative changes in the nervous system glycolipids during metamorphosis of *Xenopus laevis*, and Yates *et al.* [21] studied the patterns of brain gangliosides of *Rana catesbeiana* during metamorphosis and in the adult frog.

Lipids have the potential of serving as a source of biochemical intermediates for the tricarboxylic acid cycle. Their rapid utilization during a period of starvation usually follows when the glycogen reserves are almost depleted. Lipids have also long been recognized as important membrane constituents playing a significant role in various cellular phenomena [22–24]. Striking differences have also been reported in the fatty acids of aquatic and

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terrestrial animals [25]. Metamorphosis is a picturesque even in the life cycle of anurans involving biochemical, physiological, and anatomical alternations, which have an adaptive value in the transition from one environment to another. It follows therefore that an insight into the alternations, if any, in the lipids during metamorphosis will be interesting and hence desirable. As a first step with this view, the present author analyzed two kinds of lipids; that is, neutral lipids and phospholipids, contained in the larval tail of *Rane catesbeiana* in order to clarify the relationship between these lipid compositions and the regression of the larval tail during metamorphosis.

MATERIALS AND METHODS

Animals

Rana catesbeiana tadpoles were collected from their natural habitats in the suburbs of Ryugasaki City, Ibaragi Prefecture. The animals fed on boiled spinach in tubs in the laboratory at ca. 18–22°C. Extraction and analysis of lipids from the larval tails were made at five developmental stages of Taylor and Kollros (TK) [26]; that is, V (the length of the limb bud is twice its diameter), X (the margin of the foot paddle is indented between all five toes), XX (one or both fore-legs have protruded), XXI (the angle of the mouth has reached a point midway between the nostril and the anterior margin of the eye) and XXII-XXIII (XXII: the angle of the mouth has reached the level of the middle of the eye, XXIII: the angle of the mouth has reached the level of the posterior margin of the eyeball). Extraction and analysis of lipids from the fat bodies were made at four developmental stages of TK; that is, XV (the proximal toe pads appear), XX, XXI and XXII-XXIII. Extraction and analysis of lipids from the fat body were also mede on the adult female frog. The fat bodies of tadpoles and adult frogs, as well as the whole larval tails, were subjected to chemical analysis. After the animals were pithed, samples were removed from the body, washed in saline, and analyzed immediately.

Lipid extraction

The total lipids were extracted by our modification [27] of the Folch method [28]. The samples were homogenized with 20 volumes of chloroform/methanol (2:1 or 1:2, v/v) by the use of an ultrahomogenizer, UH-1 type, Nissei, Tokyo. The extracts were filtered through a Büchner funnel. The residues were again homogenized with 20 volumes of chloroform/methanol (2:1 or 1:2, v/v). The extracts were combined and concentrated to dryness in a rotary evaporator under nitrogen. The residue obtained was dissolved in chloroform/methanol (2:1, v/v) and was filtered through a glass filter (GA-100, Toyoroshi, Tokyo). The soluble material was concentrated to dryness in a rotary evaporator under nitrogen and then the dry lipid samples were weighed. The total lipids in the samples were determined gravimetrically. The dry lipid samples were dissolved in 25 ml of chloroform/methanol (2:1, v/v), flushed with nitrogen and stored at −20°C for lipid analysis.

Separation of simple lipids and phospholipids

Thin-layer plates of Silica Gel HR (Merk, Darmstadt, West Germany) were prepared according to routine procedure. Sodium carbonate impregnated plates were made by the method of Skipski *et al.* [29]. The lipid samples dissolved in chloroform/methanol (2:1, v/v) were applied with a Terumo microsyringe (MS-N25) on the activated plates. Simple lipids were separated by one-dimensional thin-layer chromatography (TLC) on layers of Silica Gel HR using hexane/diethyl ether/acetic acid (85:15:2, v/v/v) as developing solvent. Phospholipids were separated by one-dimensional TLC on layers of Silica Gel HR impregnated with sodium carbonate using chloroform/methanol/acetic acid/water (50:25:8:4 or 25:15:4:2, v/v/v/v) [29] as developing solvent. Authentic standards of the simple lipids and phospholipids (Sigma) were cochromatographed in each respective run.

Identification of simple lipids and phospholipids on the dried plates was made by exposing the plate to iodine vapour. The phospholipid spots were further identified by employing the following sprays: Dittmer and Lester's reagent [30] and Vaskovsky's modified spray [31] for general phos-

pholipids, ninhydrin (0.2% in butanol) for phospholipids containing free amino groups, Dragendroff reagent [32] for choline phospholipids, p-benzoquinone for ethanolamine, ammonium silver nitrate for inositol and mercuric oxide barium acetate for inositol. Details of these sprays and their diagnostic importance in thin-layer chromatography are critically described by Marinetti [33]. The simple lipids were identified by employing a dichromate sulfuric acid spray [34]. The detection of cholesterol and cholesterol ester was further confirmed by employing antimony trichloride spray [35].

Infrared spectra of the simple lipids and phospholipids were measured by pressing a film between NaCl plates.

Separation of phospholipid spots and estimation of phospholipids

Phospholipids were fractionated by one-dimensional TLC as described above. Segments of the plate containing each phospholipid were scraped off and the amount of each phospholipid was determined by measuring the phosphorus content, according to the method of Bartlett [36].

Separation of simple lipid spots and estimation of simple lipids

Simple lipids were separated by one-dimensional TLC as described above. The segment of the Silica Gel HR layer containing each simple lipid was scraped off and the amount was measured. The amount of triglyceride was estimated according to the method of Snyder and Stephens [37]. The free fatty acid isolated by the preparative Silica Gel HR layer was esterified with 3% hydrogen chloride-methanol at 100°C for three hours. The fatty acid methyl esters were analyzed and estimated at 160°C by a Shimadzu GC-5A unit equipped with a 1.5 m×3 mm glass column packed with 15% ethylene glycol succinate on Celite 545 HMDS. Methyl heptadecanoate (Applied Science Laboratories Inc., Lot 1933, Penna.) was used as the internal standard. The amount of cholesterol and cholesterol ester was estimated according to the method of Zak [38].

For confirmation of results, the thin-layer chromatographic separations and the assays of phospholipids and simple lipids were carried out in triplicate sets of tadpoles in batches as described above.

RESULTS

Lipid content in larval tail

The lipid content from the whole tail of each specimen at various stages of metamorphosis (TK stage XX to XXII-XXIII) of tadpoles is recorded in Table 1. The total lipid content at TK stage XX was about 5.44 of wet tissue and 52.05 mg/g of tissue residue after lipid extraction, and gradually increased during metamorphosis. The content was found to be about 11.13 mg/g wet tissue and 77.02 mg/g residue at TK stages XXII-XXIII.

Thin-layer chromatograms of total lipid from larval tial

Thin-layer chromatographic patterns of total lipid from the whole tails of each specimens at the various stages of premetamorphic stage (TK stages V and X) and metamorphic climax stage (TK stage XX to XXII-XXIII) of tadpoles, as shown in Figure 1, quantitatively indicate that the total

Table 1. The content of total lipids from the metamorphosing tadpole tail of *Rana catesbeiana*

	Developmental stages of Taylor and Kollros		
	X	XXI	XXII
Total lipid			
mg/g of wet tissue weight	5.44±0.10	6.88±1.22	11.13±0.46
mg/g of residue of the tissue after lipid extraction	52.05±1.11	67.20±7.65	77.02±5.60

The data are expressed as mg of lipid per g of wet tissue and per g of residue of the tissue after lipid exrraction. The values are the mean±SD of triplicate specimens.

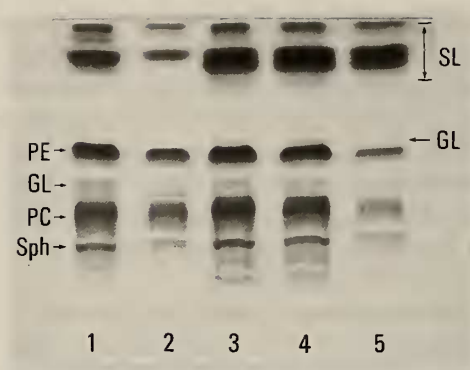

FIG. 1. Thin-layer chromatograms of total lipids from the tadpole tail of *Rana catesbeiana* at the following five developmental stages of Taylor and Kollros (TK); *i.e.* 1(V), 2(X), 3(XX), 4(XXI) and 5(XXII-XXIII). Total lipids were separated by one-dimensional thin-layer chromatography (TLC) on a layer of Silica Gel HR using chloroform/methanol/water (65:25:4, v/v/v) as developing solvent. The bands are the lipids, visualized by spray application of anthrone-sulfuric acid. SL, simple lipid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sph, sphingomyelin; GL, glycolipid.

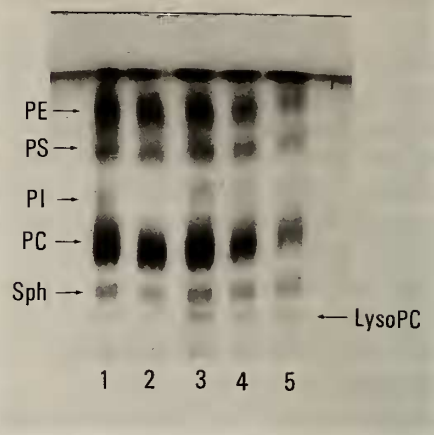

FIG. 2. Thin-layer chromatograms of total phospholipids from the tadpole tail of *Rana catesbeiana* at the following five developmental stages of TK; *i.e.* 1(V), 2(X), 3(XX), 4(XXI) and 5(XXII-XXIII). Total phospholipids were separated by one-dimensional TLC on a layer of Silica Gel HR impregnated with sodium carbonate using chloroform/methanol/acetic acid/water (50:25:8:4 or 25:15:4:2, v/v/v/v) [29] as developing solvent. The phospholipids are visualized by spray application of 50% aqueous sulfuric acid. PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; Sph, sphingomyelin; LysoPC, lysophosphatidylcholine.

lipids consist mainly of simple lipids and phospholipids as the major component, and glycolipids which were positively visualized by spraying with anthrone-sulfuric acid. By comparing these chromatographic patterns, it was found that the simple lipid fraction which contains free fatty acid (FFA) and triglyceride (TG) was quantitatively different at TK stage XX to XXII-XXIII than those at TK stages V and X.

Thin-layer chromatograms of phospholipids from larval tail

Thin-layer chromatographic separation of the phospholipids at the various stages of development of tadpoles, as shown in Figure 2, quantitatively indicates that the total phospholipids consist mainly of phosphatidylethanolamine (PE), phosphatidylcholin (PC), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (Sph), as the major components and lysophosphatidylcholin (LysoPC). A quantitative comparison shows that PC and PE were predominant, whereas PS, Sph and PI were present in lesser concentrations and LysoPC occurred in trace amount. Exept for the minor quantitative changes, the gross patterns of the phospholipids did not show any significant changes during the various developmental stages of the tadpoles.

Thin-layer chromatograms of simple lipids from larval tail

Thin-layer chromatographic separation of the simple lipids of the various developmental stages of the tadpoles, as shown in Figure 3, indicates that the total simple lipids consist mainly of TG, FFA, cholesterol (Chol) and cholesterol ester (Chol.E). By comparing each chromatographic patterns, it was found that the gross patterns of Chol and Chol.E did not show any significant changes at the various developmental stages. However, FFA and TG did show a qualitative change. Comparing gross patterns of FFA; qualitatively FFA was predominant at TK stage XX to XXII-XXIII, although FFA occurred in relatively low concentration at TK stages V and X.

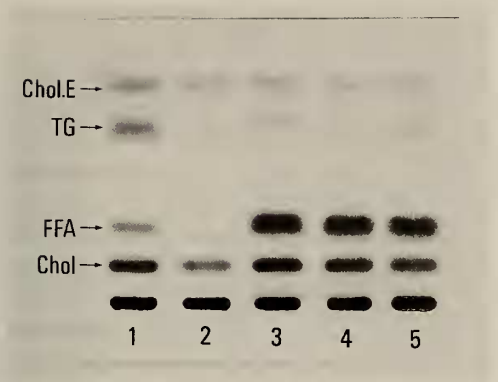

FIG. 3. Thin-layer chromatograms of total simple lipids from the tadpole tail of *Rana catesbeiana* at the following five developmental stages of TK; *i.e.* 1(V), 2(X), 3(XX), 4(XXI) and 5(XXII-XXIII). Total simple lipids were separated by one-dimensional TLC on a layer of Silica Gel HR using hexane/diethyl ether/acetic acid (85 : 15 : 2, v/v/v) as developing solvent. The bands are the lipids, visualized by spray application of 50% aqueous sulfuric acid. Chol. E, cholesterol ester; TG, triglyceride; FFA, free fatty acid; Chol, cholesterol.

Thin-layer chromatograms of simple lipids from blood, liver and muscle of larvae and adult frogs

Thin-layer chromatographic separation of the simple lipids from the blood, liver and muscle of the metamorphic climax stage (TK stage XXI) of tadpoles and adult frog in *Rana catesbeiana* qualitatively indicates that total simple lipids consist mainly of TG, FFA, Chol and Chol.E (Fig. 4). In liver and muscle tissue, TG and FFA qualitatively were predominant in metamorphic climax stage (TK stage XXI) of tadpoles, whereas they present in lesser concentration in the adult frog. FFA occurred in trace amount in the blood at both animal stages.

Thin-layer chromatograms of simple lipids from fat bodies of tadpoles

Thin-layer chromoatographic patterns of total simple lipids from a whole fat body during a period when the growth rate was reduced and metamorphic change accelated to give rise to the metamorphic climax stage (TK stage XX, XXI and XXII-XXIII) of the tadpoles indicate that a large TG faction is present in total simple lipids at each stage (Fig. 5).

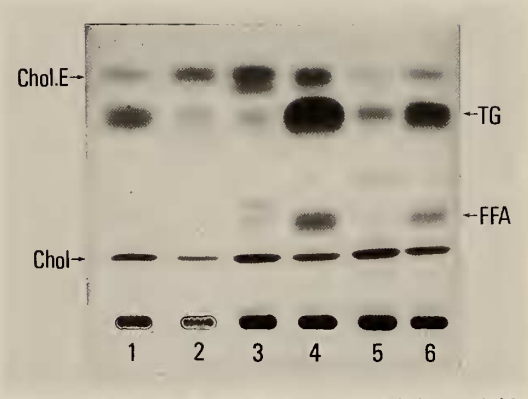

FIG. 4. Thin-layer chromatograms of total simple lipids from the blood, liver and muscle in metamorphosing tadpoles at TK stage XXI and in the adult frog of *Rana catesbeiana*. Total simple lipids were separated by one-dimensional TLC on a layer of Silica Gel HR using hexane/diethyl ether/acetic acid (85 : 15 : 2, v/v/v) as developing solvent. The bands are the lipids, visualized by spray application of 50% aqueous sulfaric acid. 1, adult frog blood; 2, tadpole blood; 3, adult frog liver; 4, tadpole liver; 5, adult frog muscle; 6, tadpole muscle. Abbreviations are described in Fig. 3.

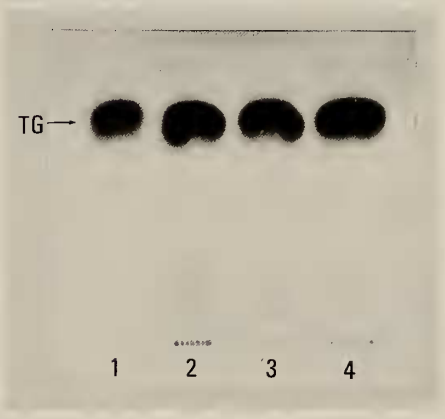

FIG. 5. Thin-layer chromatograms of total simple lipids from the fat body of *Rana catesbeiana* at following four developmental stages of TK; *i.e.* 1(XV), 2(XX), 3(XXI) and 4(XXII-XXIII). Total simple lipids were separated by one-dimensional TLC on a layer of Silica Gel HR using hexane/diethyl ether/acetic acid (85 : 15 : 2, v/v/v) as developing solvent. The bands are the lipids, visualized by spray application of 50% aqueous sulfuric acid. TG, triglyceride.

Thin-layer chromatograms of simple lipids from fat bodies of adult females

Thin-layer chromatographic patterns of total

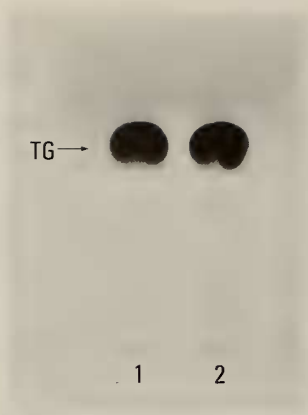

FIG. 6. Thin-layer chromatograms of total simple lipids from the fat body in duplicate mature female specimens (1, 2) of *Rana catesbeiana*. Total simple lipids were separated by one-dimensional TLC on a layer of Silica Gel HR using hexane/diethyl ether/acetic acid (85:15:2, v/v/v) as developing solvent. The bands are the lipids, visualized by spray application of 50% a aqueous sulfuric acid. TG, triglyceride.

simple lipids from the whole fat body of the mature female *Rana catesbeiana* qualitatively indicate that a relatively large amount of TG is present at each of various stages of development, as described above (Fig. 6).

Quantitative data of lipid classes

The alternations in various components of phospholipids and simple lipids are shown in Table 2. The quantitative data of phospholipid classes, PE, PS, PI, PC, Sph and LysoPC, at the 5 developmental stages of Taylor and Kollros (TK), V, X, XX, XXI and XXII-XXIII, were obtained by the routine method previously described. The quantitative data of simple lipid classes, FFA, TG, Chol and Chol.E, at the same five developmental stages were also obtained following the same routine method.

The total simple lipid content of total lipid remained essentially unchanged between TK stages V and X at ca. 33% during development. It gradually increased during metamorphic climax stage, becoming about 70% at TK stages XXII-XXIII.

The quantitative data for the total phospholipid amount in total lipid also remained unchanged between TK stages V and X, at ca. 66%, during development. However, in contrast with the quantitative data for the simple lipid fraction, the data for total phospholipid gradually decreased during metamorphosis and the content was found to be ca. 29% at TK stages XXII-XXIII. During meta-

TABLE 2. Analysis of the lipid composition from the tadpole tail during metamorphosis of *Rana catesbeiana*

	Developmental stages of Taylor and Kollros				
	V	X	XX	XXI	XXII-XXIII
Total simple lipid (% total lipid)	32.6±2.8	33.0±3.1	55.4±3.4	60.3±2.3	70.1±3.1
Total phospholipid (% total lipid)	66.6±3.4	66.1±2.3	43.3±1.5	38.5±1.2	28.7±1.9
Simple lipid classes (% total lipid)					
Free fatty acid	15.1±0.6	12.3±0.5	30.4±1.6	29.8±1.3	38.3±1.4
Cholesterol	10.1±0.4	12.8±0.7	11.8±0.6	14.2±0.5	13.1±0.5
Triglyceride	3.5±0.2	4.2±0.2	9.2±0.6	11.7±0.6	13.2±0.7
Cholesterol ester	3.9±0.3	3.7±0.2	4.0±0.3	4.6±0.4	5.5±0.4
Phospholipid classes (*% total phospholipid*)					
Phosphatidylcholine	45.6±0.5	48.2±1.2	45.1±2.1	43.7±0.8	41.2±1.8
Phosphatidylethanolamine	33.2±0.9	30.0±1.3	33.8±1.4	31.3±0.5	28.3±1.4
Phosphatidylserine	7.9±0.7	5.0±0.2	4.3±0.8	5.5±0.6	5.0±0.7
Phosphatidylinositol	2.7±0.6	4.0±0.5	4.0±0.6	4.6±0.4	4.2±0.5
Sphingomyelin	7.6±0.5	7.3±0.4	8.1±0.8	9.8±0.6	13.7±0.9
Lysophosphatidylcholine	3.0±0.3	5.5±0.2	4.7±0.5	5.2±0.5	7.6±0.8

The data are expressed as the weight percentage. The values are mean±SD of triplicate specimens.

morphosis, there was a compensatory relationship in amount between the simple lipid and phospholipid fraction.

As shown in Table 2, a large amount of free fatty acid was detected. The weight percentage of free fatty acid in total lipid was about 12 to 15% during the premetamorphic stage from TK stages V and X increased dramatically to about 30 to 38% during the metamorphic climax stage from TK stage XX to XXII-XXIII. The weight percentage of triglyceride was about 4% at the premetamorphic stage from stage TK V to X. Though the ratio of triglyceride in total lipid was not as large as that of free fatty acid, the relative percentage of both substances dramatically increased after TK stage XX. The ratio of cholesterol to total lipid was approximately 10 to 13% at TK stages V and X, and did not significantly change at TK stage XX to XXII-XXIII. Cholesterol ester was detected at each TK stage from V to XXII-XXIII: the ratio of cholesterol ester to total lipid was about 4 to 6%, and significant changes were not detected throughout the metamorphisis. Phosphatidylcholine and phosphatidylethanolamine were the major components of phospholipid classes. Phosphatidylcholine occupied about 41 to 48% of total phospholipids, while phosphatidylethanolamine occupied about 28 to 34% throughout TK stage V to XXII-XXIII. The ratio of phosphatidylcholine and phosphatidylethanolamine to total phospholipids was relatively stable. The ratio of sphinogomyelin to total phospholipids did not change during TK stage V to X, while afterward it increased slightly until TK stages XXII-XXIII. The ratio of phosphatidylserine, phosphatidylinositol and lysophosphatidylcholine did not significantly change from TK stage V to XXII-XXIII.

DISCUSSION

From the present study, it is evident that glycerol-based phospholipids including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol are predominantly present among the lipids in larval tissues and in eucaryotic cell membranes [24, 39] (Fig. 2, Table 2). Sphingomyelin (sphingosine-based lipids) and cholesterol are also major components of the cell membrane. In this animal, triglycerides may also play roles in energy storage as described for other animals [40] in whose adipose tissue they are present (Figs. 4, 5, 6). Since many biochemical alternations that occur during the metamorphosis of anuran tadpoles have been shown to be influenced by thyroxine [1, 3, 5, 6, 41], significant alternations in lipids during growth and metamorphosis may also be reasonably considered to take place under its influence. Thyroid hormone acts directly on each type of cell to induce two different events; death of epidermal cells, mesenchymal fibroblasts and probalby muscle cells, and activation of macrophages during metamorphosis [5, 6].

Lipid seems to be an important constituent of the tadpole body and the values for percentage total lipids and neutral lipids also increase during pre- and prometamorphosis; this is followed by significant decease during the metamorphic climax. Phospholipids, on the other hand, apparently do not undergo significant variation on a percentage body weight basis, though during larval development, increase in their amounts is discernible [19]. Urbani [17] observed a fourfold decrease in total lipid content during metamorphosis. Sawant and Varute [19] showed such decrease to be due to that in various constituents of neutral lipids and phospholipids.

During the climax period, when tadpoles do not feed and histolytic events such as degeneration of internal gills, skin degeneration in fore limb window formation and tail regression occur maximally, lipid content decrease sharply, especially that of neutral lipids such as triglycerides that accumlate during development and endogenous energy alone is available.

The decrease in total phospholipids during metamorphosis may quite likely be due to the breakdown of tail cell membranes whose phospholipids form integral components in the histolytic events of metamorphosis. This is supported by the fact that fatty acids are not present in the early stages of metamorphosis. The catabolic breakdown of phospholipids leads to the formation of fatty acids and nitrogenous bases [23]. Phospholipid degradation in *E. coli* has been summarized by Rock and Cronan [42] as follows. Phospholipid

degradation, i.e., the hydrolysis of fatty acids from the 1-position and 2-position of phospholipids, is hydrolyzed by phospholipase A, and in particular, the hydrolysis of fatty acids from the 1-position occurs most rapidly in the cells of *E. coli*. Phospholipase A is located in the soluble fraction (cytoplasm) and outer cell membranes of *E. coli*. Lysophospholipase is located in the soluble fraction and inner cell membranes of *E. coli*. The biochemical and physiological functions of these degradative enzymes remain unknown in amphibian cells during development and metamorphosis. However, a similar phenomenon of phospholipid degradation by these enzyme in macrophages may possibly occur during the regression of tadpole tails in *Rana catesbeiana*.

The present study demonstrates that there may possibly be a correlation between the regressive process of this tadpole tail and the presence of relatively large amounts of free fatty acids during metamorphosis. It would thus follow these free fatty acids may mainly be those hydrolyzed from the 1-position and 2-position of phospholipids during metamorphosis. It is generally known that the transport of fatty acids is a major function of triglycerides in mammals [40]. In this animals, free fatty acids may be stabilized by association with albumin or similar proteins, and albumin or similar proteins may carry fatty acids from the tail to the liver or fat body. These fatty acids may be regarded as triglyceride components in a fat body which is produced in this tadpole during metamorphosis. Triglycerides may play major roles in energy storage as deposits of adipose tissue. Additional research should be conducted for greater clarification of the functions of phospholipid degradative enzymes in the regressive process of tadpole tails during metamorphosis.

Quantitative and qualitative data on glycolipids in the tadpole tail during metamorphosis in *Rana catesbeiana* will be reported in the next publication.

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